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Multiplex PCR: Optimization and Application in Diagnostic Virology

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INTRODUCTION

During the past decade, advances in PCR technology and other DNA signal and target amplification techniques have resulted in these molecular diagnostics becoming key procedures (4, 107, 117). Such techniques are conceptually simple, highly specific, sensitive, and amenable to full automation (54, 115). The most mature of these technologies, PCR, is in one variant or another now common in research laboratories and is used increasingly in routine diagnostic laboratory settings and undergraduate and high-school teaching (32, 38, 40, 101). In diagnostic laboratories the use of PCR is limited by cost and sometimes the availability of adequate test sample volume. To overcome these shortcomings and also to increase the diagnostic capacity of PCR, a variant termed multiplex PCR has been described. In multiplex PCR more than one target sequence can be amplified by including more than one pair of primers in the reaction. Multiplex PCR has the potential to produce considerable savings of time and effort within the laboratory without compromising test utility. Since its introduction, multiplex PCR has been successfully applied in many areas of nucleic acid diagnostics, including gene deletion analysis (19, 20), mutation and polymorphism analysis (86, 96), quantitative analysis (94, 124), and RNA detection (51, 126). In the field of infectious diseases, the technique has been shown to be a valuable method for identification of viruses, bacteria, fungi, and/or parasites. A representative list of such agents is shown in Table 1.

Based upon our own experience with multiplex PCR and those of other authors appearing in the literature during the last 10 years, we review the theoretical and practical basis of the development and optimization of multiplex PCR systems and discuss the application and potential of this technique in the field of diagnostic virology.

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PRINCIPLE AND DEVELOPMENT OF MULTIPLEX PCR

A number of review and research articles have provided detailed descriptions of the key parameters that may influence the performance of standard (uniplex) PCR (17, 57, 88, 91, 112). Fewer publications discuss multiplex PCR (18, 28, 43).

Primers and Multiplex PCR Efficiency

The first few rounds of thermal cycling have substantial effect on the overall sensitivity and specificity of PCR (92). Assuming efficient denaturation of the target, overall success of specific amplification depends on the rate at which primers anneal to their target and the rate at which annealed primers are extended along the desired sequence during the early, middle, and late cycles of the amplification. Factors preventing optimal annealing rates include poorly designed primers and suboptimal buffer constituents and annealing temperature. The extension rate of specific primer-target hybrids depends on the activity of the enzyme, availability of essential components such as deoxyribonucleoside triphosphates (dNTPs), and the nature of the target DNA. Thus, the majority of modifications to improve PCR performance have been directed towards the factors affecting annealing and/or extension rates.

The optimization of multiplex PCRs can pose several difficulties, including poor sensitivity or specificity and/or preferential amplification of certain specific targets (76). The presence of more than one primer pair in the multiplex PCR increases the chance of obtaining spurious amplification products, primarily because of the formation of primer dimers (9). These nonspecific products may be amplified more efficiently than the desired target, consuming reaction components and producing impaired rates of annealing and extension. Thus, the optimization of multiplex PCR should aim to minimize or

TABLE 1. Representative list of applications of multiplex PCR to the diagnosis of infectious diseases

Infectious agent	Pathogens targeted	Clinical manifestation(s) and/or specimen	Reference(s)
Virus	HIV-1, HIV-2, HTLV-1, and HTLV-2	Blood	45
	HSV-1, HSV-2, VZV, CMV, HHV-6, EBV, and EVs[a]	Meningitis, encephalitis, or meningoencephalitis; CSF	13, 14
Bacterium	*Haemophilus influenzae*, *Streptococcus pneumonia*, *Mycoplasma catarrhalis*, and *Alloiococcus otitidis*	Upper respiratory tract	42
	Campylobacter jejuni and *Campylobacter coli*	Human campylobacteriosis	37
	Actinomyces actinomycetemcomitans, *Porphyromonas intermedia*, and *Porphyromonas gingivalis*	Periodontal infection	34
	N. gonorrhoeae and *C. trachomatis*	Genital infections	60, 118
	C. trachomatis, *N. gonorrhoeae*, *Ureaplasma urealyticum*, and *M. genitalium*	Genital infections	59
Parasite	*Giardia lamblia* and *Cryptosporidium parvum*	Diarrheal disease; water	52, 89
	Leishmania spp.	Leishmaniasis	5, 39
Combination	HSV, *H. ducreyi*, and *T. pallidum*	Genital ulcer disease	7, 73
	HPVs, HSV, and *C. trachomatis*	Genital swabs	64
	Adenovirus, HSV, and *C. trachomatis*	Keratoconjunctivitis	Yeo et al.[b]
	EV, influenza viruses A and B, RSV, PIV types 1 and 3, adenovirus, *M. pneumoniae*, and *C. pneumoniae*	Acute respiratory tract infections	36

[a] EVs, enteroviruses.
[b] Abstr. 97th Gen. Meet. Am. Soc. Microbiol., 1997.

reduce such nonspecific interactions. Empirical testing and a trial-and-error approach may have to be used when testing several primer pairs, because there are no means to predict the performance characteristics of a selected primer pair even among those that satisfy the general parameters of primer design (43). However, special attention to primer design parameters such as homology of primers with their target nucleic acid sequences, their length, the GC content, and their concentration have to be considered (26, 65, 72, 88, 95, 120). Ideally, all the primer pairs in a multiplex PCR should enable similar amplification efficiencies for their respective target. This may be achieved through the utilization of primers with nearly identical optimum annealing temperatures (primer length of 18 to 30 bp or more and a GC content of 35 to 60% may prove satisfactory) and should not display significant homology either internally or to one another (17, 26, 43).

Preferential amplification of one target sequence over another (bias in template-to-product ratios) is a known phenomenon in multiplex PCRs that are designed to amplify more than one target simultaneously (68, 76, 109). Based on both theoretical modeling and experimental studies, two major classes of processes that induce this bias have been identified, PCR drift and PCR selection (108). PCR drift is a bias assumed to be due to stochastic fluctuation in the interactions of PCR reagents particularly in the early cycles, which could arise in the presence of very low template concentrations (26, 68); variations in the thermal profiles of a thermocycler, resulting in unequal ramping temperatures; or simple experimental error. PCR selection, on the other hand, is defined as a mechanism which inherently favors the amplification of certain templates due to the properties of the target, the target's flanking sequences, or the entire target genome. These properties include interregion differences in GC content, leading to preferential denaturation; higher binding efficiency because of GC-rich primers; differential accessibility of targets within genomes due to secondary structures; and the gene copy number within a genome. In addition, the choice of primers has been shown to be crucial to avoid PCR selection. Amplification biases that were strongly dependent on the choice of primers and dependent to a lesser extent on the templates have been described (100). Some primer pairs with high amplification efficiency resulted in templates being saturated (plateau phase), while other primer pairs produced product independent of starting template concentrations. Primers with lower amplification efficiency resulted in product concentrations below the saturation concentrations, and depending on the template, either the expected product ratio or bias was observed.

Other PCR Components

Alteration of other PCR components such as PCR buffer constituents, dNTPs, and enzyme concentrations in multiplex PCR over those reported for most uniplex PCRs usually results in little, if any, improvement in the sensitivity or specificity of the test. Increasing the concentration of these factors may increase the likelihood of mis-priming with subsequent production of spurious nonspecific amplification products. However, optimization of these components in multiplex PCRs that are designed for simultaneous amplification of multiple targets may prove beneficial. For example, in the multiplex PCR for the dystrophin gene (nine genomic targets), a *Taq* DNA polymerase concentration (with an appropriate increase in $MgCl_2$ concentration) four to five times greater than that required in uniplex PCR was necessary to achieve optimal nucleic acid amplification (19). Variation in concentrations of reaction components above those used in uniplex PCR probably reflects the competitive nature of the PCR process. The desired target DNA can be outcompeted by the more efficient amplification of other targets (including nonspecific products), leading to decreases in the efficiency of the amplification of the desired targets and hence sensitivity of the reaction (79).

PCR additives, such as dimethyl sulfoxide, glycerol, bovine serum albumin, or betaine, have been reported to be of benefit in multiplex PCRs (49, 62). The components may act to prevent the stalling of DNA polymerization, which can occur through the formation of secondary structures within regions of template DNA during the extension process (44). Such cosolvents may also act as destabilizing agents, reducing the melting temperature of GC-rich sequences, or as osmopro-

tectants, increasing the resistance of the polymerase to denaturation (44, 83).

Variations in Methodology To Improve Sensitivity and Specificity

A straightforward solution to difficulties encountered in the development of multiplex PCR has been the use of hot start PCR (21) and/or nested PCR (123). The former often eliminates nonspecific reactions (particularly production of primer dimers) caused by primer annealing at low temperature (4 to 25°C) before commencement of thermocycling (21). The procedure has recently been made more practicable through the use of a nonmechanical hot start methodology which involves the use of a form of *Taq* polymerase, for example, Amplitaq Gold (Roche Diagnostics), which is activated only if the reaction mixture is heated at approximately 94°C for 10 min (the first denaturation step) (8, 53). Nested PCR increases the sensitivity and specificity of the test through two independent rounds of amplification using two discrete primer sets. Although this adaptation is undoubtedly effective in most cases, it also considerably complicates the practical application of PCR. The second round of amplification delays results, increases the possibility of cross-contamination, and may complicate automation.

General Considerations for Multiplex PCR Development

Development of multiplex PCRs should follow a rational approach for the inclusion or exclusion of specific pathogens in the assay. These pathogens can be organ system specific or symptom specific with respect to the age of the patient and the epidemiological characteristics of these pathogens. PCR conditions, such as compatibility among the primers within the reaction mixture such that there is no interference, is of great technical importance. The primer pairs must be inclusive for as many strains of the target pathogen as possible, and depending on the amplicon detection method, their targets are easily resolvable. The latter may be achieved by using primer pairs that result in PCR products that can be separated and clearly visualized using gel electrophoresis or hybridization probes with maximum specificity. Prior to application in a clinical setting, multiplex PCRs must be evaluated for their sensitivity as compared with their corresponding uniplex PCRs using both serial dilutions of the target DNA and clinical specimens.

Wherever possible, multiplex PCRs should avoid the use of nested primers requiring a second round of amplification. The latter is a major contributor to false-positive results due to carryover contamination, although anticontamination protocols including PCR controls (reaction and specimens extraction controls) must be implemented in all PCR-based protocols (55). Likewise, precautions and methodologies to avoid false-negative results due to reaction failure have to be considered (104). Multiplex PCRs that amplify target sequences along with the presence of external or internal control target nucleic acids to indicate reaction failure have been developed (49, 62, 69).

APPLICATION OF MULTIPLEX PCR IN DIAGNOSTIC VIROLOGY

During the last decade, a number of studies have demonstrated the practicality of identifying viral pathogens in many clinical and epidemiological settings using multiplex PCR (Table 2). The technique has been used to screen for individual or symptom-associated viruses and examine associations of virus infection with disease. In addition, the technique has been shown to be a powerful and cost-effective tool for typing and subtyping virus strains in different epidemiological studies.

Neurotropic Viruses

PCR has proved to be a powerful tool for investigating meningitis and encephalitis caused by a variety of viruses. In neurological disease the requirement of rapid and reliable diagnosis to provide a rational basis for chemotherapy and limit unnecessary procedures and irrelevant therapy has driven development. The wide range of viruses associated with neurological disease includes herpes simplex virus (HSV); cytomegalovirus (CMV); varicella-zoster virus (VZV); Epstein-Barr virus (EBV); human herpes virus 6 (HHV-6); the enterovirus group, including echoviruses, polioviruses, and coxsackieviruses; adenoviruses; JC and BK viruses; arenaviruses; paramyxoviruses; rabies; and arboviruses. In view of the large number of potentially neuroinvasive viruses and because of the limited volume of the most useful diagnostic specimen—cerebrospinal fluid (CSF)—a number of multiplex PCRs have been developed (12–15, 82, 102).

The feasibility of simultaneous screening for viruses, bacteria, and parasites in CSF specimens from patients with aseptic meningitis or encephalitis has been described (82). This study by Read et al. (82) utilized three nested multiplex PCRs for detection of HSV and VZV; EBV and HHV-6; and members of the enterovirus group and echovirus type 22 and 23. In addition, two uniplex PCRs were used for detection of CMV and JC virus. In a total of 2,233 CSF specimens from 2,162 patients, the PCR was positive in 147 specimens from 143 patients (6.6% of all patients) including enteroviruses (77 patients), HSV-1 (20 patients), VZV (7 patients), HSV type 2 (HSV-2) (6 patients), CMV (3 patients), JC virus (2 patients), and HHV-6 (1 patient). All PCR assays remained negative with 28 control CSF specimens. The clinical sensitivity and specificity of this PCR were not determined because full clinical information was not available for all of the patients.

A nested multiplex PCR for detection and differentiation of HSV-1 and -2 on the basis of PCR product size has also been described (14). In a prospective analysis, a total of 417 CSF specimens obtained from 395 consecutive patients with clinical suspicion of HSV encephalitis, meningitis, or meningoencephalitis were tested by multiplex PCR. The test was positive for HSV-1 in 11 specimens (2.6%) from 10 patients and for HSV-2 in 4 specimens (1.0%) from 3 patients; no coinfection with both types was reported. The same multiplex PCR was used to test a total of 178 CSF samples obtained from 171 patients with clinical suspicion of herpes virus infection (15). The assay was positive for HSV-1 in three samples (1.7%) from two patients (1.2%) and for HSV-2 in one sample, and one patient tested positive in a nested uniplex CMV PCR. A similar procedure to detect the DNA of both viruses (HSV-1 and -2) was applied to CSF samples from 918 human immunodeficiency virus (HIV)-infected patients with neurological symptoms (22). In patients for whom a diagnosis was confirmed at autopsy, the test was positive for HSV-1 or -2 for 19 patients (2%), producing a sensitivity and specificity of 100 and 99.6%, respectively.

The first nested multiplex PCR for detection and typing of herpesviruses (HSV-1 and -2, VZV, CMV, HHV-6, and EBV) was applied to CSF from patients with meningitis, encephalitis, and other clinical syndromes (102). By utilizing equimolar concentrations of primers aligning the 3′ ends with one of two consensus regions within the herpesvirus DNA polymerase gene and the 5′ ends with the related or nonrelated sequences of each agent to be amplified, the first round of amplification yielded a 194-bp fragment indicating the presence of herpes-

TABLE 2. Application of multiplex PCR for diagnosis of viral infections

Clinical manifestation(s)	Specimen(s)	Viruses and/or other agent(s) targeted	Reference(s)
Meningitis, encephalitis, and/or meningo-encephalitis	CSF	HSV-1, HSV-2, and CMV	14
		HSV and VZV; EBV and HHV-6	81
		HSV-1, HSV-2, VZV, CMV, HHV-6, and EBV	102
		HSV-1 and HSV-2	15
		HSV-1, HSV-2, VZV, CMV, HHV-6, EBV, and EVs[a]	12, 13
		CMV, EBV, HHV-6, HHV-7, and HHV-8	77
		EBV and *T. gondii*	87
Upper and lower respiratory infections	Throat, nose, and nasopharyngeal swabs; nasopharyngeal and endotracheal aspirates; bronchoalveolar lavage	Influenza viruses A and B	30
		PIV types 1, 2, and 3	27
		Influenza virus and RSV	98
		RSVs A and B, influenza viruses A and B, PIV types 1, 2, and 3	33
		RSV, PIVs, adenovirus	74
		EV, influenza viruses A and B, RSV, PIV types 1 and 3, adenovirus, *M. pneumoniae*, *C. pneumoniae*	36
Conjunctivitis, keratitis, keratoconjunctivitis	Conjunctival and corneal swabs	HSV-1 and HSV-2	15
		Adenovirus and HSV	49
		Adenovirus, HSV, and *C. trachomatis*	Yeo et al.[b]
Genital ulcer disease	Genital ulcer swabs	HSV, *H. ducreyi*, and *T. pallidum*	7, 66, 73
Genital lesions	Lesion and endocervical swabs	HPVs, HSV, and *C. trachomatis*	64
HPV-associated genital disease	Cervical scrapings, smears, and biopsies; vaginal and vulval swabs	HPVs	23, 35, 56, 75, 97, 123
Vesicular rashes	Vesicle fluids	HSV and VZV	3
Hepatitis	Serum and plasma samples	HBV genotypes	84
		HCV, HGV, and GB viruses	16
Immunocompromised status	Plasma	HHV-6 and HHV-7	67
	Blood	HIV-1, HIV-2, HTLV-I, HTLV-2	45

[a] EVs, enteroviruses.
[b] Abstr. 97th Gen. Meet. Am. Soc. Microbiol., 1997.

virus. The second round of amplification utilizing primer mixtures contained nonhomologous and type-specific primers selected from different regions of the aligned DNA polymerase genes of human herpesviruses produce a product with a different size for each related virus. The method amplified the corresponding virus in infected cells and in five clinical samples (HSV-1 PCR-positive CSF from a patient with encephalitis, HSV-2 PCR-positive CSF from a patient with meningitis, VZV culture-positive vesicular fluid from a patient with shingles, CMV culture-positive urine from a congenitally infected patient, and EBV PCR-positive peripheral blood from a patient with a lymphoproliferative syndrome). In addition, the use of primers targeting consensus regions may allow recognition of new, undescribed human herpesviruses. The detection of a 194-bp fragment after the first reaction with no positive signal in the second round of amplification could reflect the detection of a new human herpesvirus. The test was further modified to include a reverse transcription step and primer pairs to detect enterovirus cDNA (12). This PCR was then evaluated in 21 patients with etiologically well-characterized aseptic meningitis and encephalitis. HSV DNA was detected in nine patients, VZV DNA was detected in 6 patients, and enterovirus RNA was detected in 6 patients. The test was further evaluated for detection of these same viruses in CSF samples by a prospective study of 200 neurological-disease patients suspected to have viral infections. Enterovirus was detected in 49 patients, HSV was detected in 3 patients, VZV was detected in 6 patients, CMV was detected in 12 patients, EBV was detected in 2 patients, CMV and HSV were detected in one AIDS patient with encephalitis, and CMV and EBV were detected in another AIDS patient with polyradiculomyelitis. For detection of echovirus 30 in 50 patients with aseptic meningitis, the multiplex reverse transcription (RT)-PCR was more sensitive (90% sensitivity) than cell culture (26% sensitivity) and the Amplicor EV test (86% sensitivity). These studies demonstrate the utility of this multiplex RT-PCR for detection of enteroviruses and herpesviruses in CSF samples from patients with various neurological manifestations and the usefulness of the technique in patient management and design of antiviral therapy.

In the United Kingdom, a nested multiplex PCR for the detection of HSV-1 and -2, VZV, and enteroviruses, the four most common causes of viral meningitis and encephalitis, was developed and evaluated using a total of 1,683 consecutive CSF samples (81). The test was positive in 138 (8.2%) of the specimens (enteroviruses in 51 samples, HSV-2 in 33 samples, VZV in 28 samples, and HSV-1 in 25 samples). Of the 51 patients positive for enterovirus RNA, 17 were babies less than 6 months old in whom the CNS infection was detected as part

of a general infection screen and 34 patients were older children and adults who had encephalitis and meningitis. In the group positive for VZV (28 patients), 16 patients had meningitis and 10 had encephalitis but clinical details were not available for 2 patients. HSV-1 was detected in two babies less than 6 months old and in 23 adults (22 had encephalitis and 1 had a benign lymphocytic meningitis). The HSV-2-positive patients (33 patients) included five babies less than 6 months old, two adults with meningoencephalitis, and 26 patients more than 6 months old with benign lymphocytic meningitis. These tests proved suitable for routine use in a diagnostic laboratory and highlighted the importance of screening for more than one virus in patients with meningitis and encephalitis.

Although the studies described above (13–15, 81, 82, 102) produced satisfactory results in terms of simultaneous screening for neurological manifestation-associated viruses (for example herpesviruses), the multiplex PCRs developed utilized a nested strategy. The latter as described earlier may increase the chance of false-positive results due to contamination and may also complicate automation. A recent study (63) utilized a PCR assay which precludes the use of nested primers for simultaneous amplification of herpesviruses DNAs. This assay, termed consensus PCR, uses a pair of "stair" primers, which are based on consensus sequences selected from within the DNA polymerase gene and were 76 to 86% identical to the genomic sequences of the six herpesviruses (HSV-1, HSV-2, CMV, EBV, VZV, and HHV-6) that may infect the CNS. Each stair primer used comprised an equimolar mixture of 11 oligonucleotides corresponding to a consensus sequence: all primers had the same 5′ end but extended for 20 to 30 nucleotides in the 3′ direction. The PCR products were analyzed by hybridization in microtiter plates using virus-specific, biotinylated oligonucleotide probes. The consensus PCR was evaluated using 142 CSF samples previously tested by standard uniplex PCRs. Eighteen samples (12.7%) tested positive by the uniplex PCRs, and 37 (26%) tested positive by the consensus PCR, including 3 samples that had coinfections (CMV, VZV, and HSV-2; VZV and HSV-2; and CMV and HHV-6). Of the 142 CSF samples, 103 were classified as negative by both the uniplex PCRs and the consensus PCR. In addition, the test showed high diagnostic utility in that several cases were found to be positive for viruses for which tests were not requested by the clinician.

The problem in evaluating all of the aforementioned multiplex PCR studies is that most have not included complete patient detail. Thus, while many positive results have been related to compatible clinical illness, positive results are not reported in all patients with similar conditions. Nevertheless, the multiplex PCR detects more positive specimens and is more rapid than conventional techniques such as culture or serology. However, the latter procedures are either insensitive or slow and make an unsatisfactory yardstick ("gold standard") against which to measure the accuracy of multiplex PCR. Because uniplex PCR has more data available and thus is better substantiated as to its clinical value, results of multiplex PCR should be compared with those of uniplex PCR to ensure that multiplex PCR has equivalent sensitivity, specificity, and clinical relevance.

Respiratory Viruses

Viruses that commonly cause respiratory infection include respiratory syncytial virus (RSV), influenza viruses and parainfluenza viruses (PIV), and adenovirus, especially in infants and young children. Infection with these viruses may result in severe lower or upper respiratory tract disease requiring hospitalization. Thus, sensitive and rapid testing for these viruses is crucial to reduce the potential of nosocomial transmission to high-risk patients, limit unnecessary antibiotic use, and direct appropriate therapy following a specific diagnosis (119). For this reason, a number of studies have aimed to develop and evaluate multiplex PCR for detection of these viruses and provided substantial evidence of the utility of this technique as an important tool for management of patients presenting with respiratory infections. A number of studies have utilized multiplex PCR to both detect and type or subtype influenza viruses, PIVs, and RSV in clinical specimens and are summarized below.

A nested multiplex RT-PCR which included three primer pairs in each round of amplification was utilized for the simultaneous detection, typing, and subtyping of influenza type A (H3N2 and H1N1) and type B viruses in a prospective surveillance of influenza in England in the 1995–1996 winter season (30). A total of 619 combined nose and throat swabs from patients with an influenza-like illness were analyzed by culture and multiplex PCR. The multiplex RT-PCR detected influenza viruses in 246 (39.7%) samples compared to the 200 (32.3%) which yielded influenza viruses in culture. In addition, there was excellent correlation between the multiplex RT-PCR and culture for typing and subtyping of influenza viruses (100%) and for temporal detection of influenza A H3N2 and H1N1 viruses. It was concluded that whereas the multiplex RT-PCR demonstrated its utility in detection of influenza viruses in patients with influenza-like illness, patients with influenza-like illness who are negative for influenza viruses may harbor a pathogen(s) producing a syndrome difficult to distinguish clinically from true influenza (for example, RSV). Indeed, when this multiplex RT-PCR was modified so that it was capable of detecting and subtyping influenza A (H1N1 and H3N2) and B viruses as well as RSV subtypes A and B in respiratory clinical samples (98), the assay again demonstrated excellent (100%) correlation with the results of culture and serology. The ability of the test to detect viral coinfection in both simulated specimens and clinical samples was also demonstrated.

A nested multiplex RT-PCR using three primer pairs was developed to detect PIV types 1, 2, and 3 in throat and nasopharyngeal swabs (27). In the first round of amplification, similar-size fragments are produced. In the second round of amplification a series of three internal primer pairs are introduced, producing type-specific amplicons that were easily differentiated based on size upon gel electrophoresis. The test detected and correctly typed PIV in 15 isolates and 26 of 30 (87%) previously positive nasopharyngeal specimens but remained negative in naso- or oropharyngeal specimens and/or culture isolates of 33 unrelated respiratory tract pathogens. In a modified version, the test was also used to detect RSV and adenovirus utilizing five primer sets to amplify cDNA of RSV subtypes A and B; PIV types 1, 2, and 3; and DNA of adenovirus types 1 to 7 (74). The test was sensitive and specific for all 12 tissue culture-grown prototype viruses and when applied to respiratory specimens was more sensitive (41 of 112) than direct immunofluorescence or antigen detection following culture (34 of 112). Among positive samples, multiple respiratory viruses were found in four specimens, further illustrating the potential utility of this multiplex PCR assay.

A multiplex quantitative RT-PCR enzyme hybridization assay (Hexaplex; Prodesse, Inc., Milwaukee, Wis.) which combines primers originating from highly conserved regions of 7 respiratory viruses (RSV subtypes A and B; PIV types 1, 2, and 3; and influenza viruses A and B) with probes for the detection of PCR products using enzyme hybridization assay has also been described (33). The assay provides rapid simultaneous

detection, identification, and quantitation of these viruses in nasal wash specimens in a single test. The primer and the probes utilized were evaluated using multiple virus isolates from each group and resulted in specific PCR products from all tissue culture-positive specimens without cross-reactivity among these seven viruses or with other common human respiratory viruses. The Hexaplex assay was applied on nasal wash specimens from 69 children with signs of lower respiratory tract infection and 40 specimens from asymptomatic children. Of the 69 specimens from symptomatic children, 37 were positive by the Hexaplex assay but only 29 of these were culture positive. Both the Hexaplex assay and virus culture were negative in the 40 nasal washes from the asymptomatic children. The Hexaplex assay was 100% sensitive and 98% specific in comparison with virus culture.

Multiplex PCR in combination with a heteroduplex mobility shift assay has proved to be a valuable and cost-effective tool for monitoring the emergence of new variants or new subtypes of influenza viruses arising through the phenomena of antigenic drift and antigenic shift (125, 126). On the basis of amplicon size, the amplification assay differentiates the variable region of the hemagglutinin genes of the H1 and H3 subtypes of influenza viruses A and B and the counterpart (hemagglutinin, esterase, and fusion gene) of influenza virus C. Variants within the same type or subtype are then identified by heteroduplex mobility shift assay of the amplicons. This approach proved to be a rapid, sensitive, and reliable method for the detection and typing of influenza virus and for screening for influenza virus variants, proving capable of identifying new influenza B virus variants (126).

More recently, it has been demonstrated that multiplex PCR is a useful and rapid diagnostic tool for the management of children with acute respiratory infections (36). This simplified hot start multiplex PCR allows simultaneous screening for nine different infectious agents (enterovirus, influenza viruses A and B, RSV, PIV types 1 and 3, adenovirus, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae*). The test was evaluated using clinical samples from 1,118 children with acute respiratory infections and was positive in 395 (35%) samples. Of these, 37.5% were positive for RSV, 20% were positive for influenza A virus, 12.9% were positive for adenovirus, 10.6% were positive for enterovirus, 8.1% were positive for *M. pneumoniae*, 4.3% were positive for PIV type 3, 3.5% were positive for PIV type 1, 2.8% were positive for influenza B virus, and 0.2% were positive for *Chlamydia trachomatis*. Seasonal variations in the rates of detection of the different organisms were noted. The test demonstrated levels of concordance of 95% for RSV and 98% for influenza A virus with the data obtained by commercially available enzyme immunoassay.

Genito-Urinary Infections

The utility of multiplex PCR in diagnosis of viruses associated with genital tract infection is reflected by the numerous reports detecting and typing human papilloma viruses (HPVs). These multiplex PCRs have a variety of formats but with a common aim of detecting and typing HPVs. These include PCRs which combined primers to produce a type-specific product size (97, 103) or those that utilized degenerate primers for HPV screening and strain-specific antisense primers for simple typing (56). In an alternative protocol, three consensus primer pairs simultaneously detected high-risk (for cervical carcinoma) HPV type 16 (HPV-16) and HPV-18 and low-risk HPV-6 and HPV-11, and primer pairs for more than 40 other HPV types were developed (consensus multiplex PCR) (124). This approach was considered to be a consensus multiplex PCR assay because all the primers are consensus and are multiplexed in the same PCR mixture for simultaneous amplification. One primer pair amplifies general HPV DNA from more than 40 types, including HPV-6, -11, -16, and -18 (450-bp PCR product), indicating HPV infection. Another primer pair generates a PCR product of 307 bp if HPV-16 and -18 DNA are present in the sample (high-risk HPV infection). The third consensus primer pair results in a PCR product of 550 bp with low-risk HPV infection (HPV-6 and -11). The assay was evaluated on exfoliated cell specimens obtained from 148 healthy women, biopsied specimens from 32 patients with condyloma acuminata, and biopsied specimens from 76 patients with invasive carcinoma of the uterine cervix. The multiplex PCR was positive in 47 (31.8%) of the samples from the 148 healthy women, but most of the positive samples were in samples from women infected with HPV types other than HPV-6 and -11 or HPV-16 and -18. All samples from patients with condyloma acuminata contained HPV DNA but mainly contained HPV-6/11 (87.5%), while high-risk HPV had a low prevalence rate (6.5%). HPVs were detected in samples from 69 (90.8%) of the 76 patients with cervical carcinoma, of which high-risk HPVs accounted for 82.9%. The method proved to be a simple, economical, and reliable tool for detection of HPV infection. The simultaneous amplification of the three HPV targets should allow rapid approach for distinguishing disease-related HPV types: low-risk HPVs (HPV-6 and HPV-11) involved in genital, benign lesions, such as warts or condylomata, and high-risk HPVs (HPV-16 and HPV-18), which are frequently found in malignant lesions of the lower genital tract. This should provide valuable information for monitoring and treating patients with HPV-related lesions, although the method lacks the capability to define individual HPV types, which limits its usefulness in epidemiological investigations.

Three consensus primers (two sense primers and 1 antisense primer labeled with dinitrophenyl) were used in a multiplex PCR assay for detection and typing of oncogenic and nononcogenic HPV types (23). The amplification products were hybridized with specific labeled oligoprobes mixed in two cocktails (oncogenic and nononcogenic biotinylated HPV oligoprobes) which could then be deposited in one well of streptavidin-coated microplates. PCR products were detected with anti-dinitrophenyl monoclonal antibody and horseradish peroxidase (PCR-enzyme immunoassay). The test was evaluated in cervical scrapings from 181 patients at high risk for cervical cancer selected because of their histories of cytological and/or histological cervical and vaginal abnormalities. These patients were classified with regard to the presence of lesions in cervical scrapings as none (i.e., no lesions; $n = 137$), low grade (i.e., few lesions $n = 20$), and high grade (i.e., many lesions $n = 24$). In patients without lesions, the multiplex PCR detected nononcogenic HPV in 29 patients (18.3%) and oncogenic HPV in 44 patients (32.1%), including 21 (15.3%) patients presenting with coinfection. In the low-grade group, oncogenic HPV was detected in 12 patients (60%) and nononcogenic HPV was detected in 5 patients (25%), including 4 (20%) cases of coinfection. In the high-grade patients, oncogenic HPVs were detected in 95.8% (23 patients) with coinfection with nononcogenic HPV in 4 of these patients (16.7%). The multiplex PCR was negative in scrapings from the remaining 93 patients (85 patients without lesions, 7 patients in the low-grade group, and 1 patient in the high-grade group). The authors concluded that the test is simple and reproducible and can be automated.

Another variation (75) combined a nested multiplex PCR, utilizing primers specific for low- or high-risk HPV types with restriction endonuclease analysis. The accuracy of this ap-

proach was confirmed by examining cervical scrapings from 44 patients. HPVs were detected and typed in scrapings from 7 patients with koilocytosis, 8 patients with dysplasia or metaplasia, 3 patients with condyloma acuminata, and 18 patients with cervical invasive neoplasia but in no samples from the seven healthy controls. More recently, a simple multiplex PCR utilizing three primer pairs for amplification of HPV-16, -18, and -33 in combination with a colorimetric microplate hybridization as a post-PCR detection system has been developed (35). The system uses three type-specific capture oligonucleotides linked covalently to a single microplate well and three type-specific multibiotinylated probes for detection. The methodology was evaluated using a total of 55 cervical smears and biopsy samples from 55 women with cervical lesions, resulting in 100% correlation with the results of other PCRs using consensus primers.

A multiplex PCR that combined the detection of *C. trachomatis* and two viruses (HPV and HSV) was developed, optimized, and evaluated using cervical and endocervical specimens from patients suspected to be infected with one or more of these agents (64). The test produced 100% correlation with the results of the uniplex PCRs in 92 genital swabs (29 were positive for HSV, 16 were positive for HPVs, and one was positive for *C. trachomatis*). In addition, a coinfection with HPV and HSV was detected by the multiplex PCR. In other studies (7, 66, 73), primer pairs for HSV were combined with those for *Haemophilus ducreyi* and *Treponema pallidum* to construct a multiplex PCR for diagnosis of genital ulceration. The PCR products were detected utilizing a colorimetric detection system in which three separate microwells containing immobilized oligonucleotide capture probes were used. In one of these studies (73), the sensitivity of this multiplex PCR in 298 genital ulcer swab specimens to detect HSV, *H. ducreyi*, and *T. pallidum* were 100, 98.4, and 91%, respectively, compared to 71.8, 74.2, and 81% by HSV culture, *H. ducreyi* culture, and darkfield microscopy for *T. pallidum*, respectively. The same multiplex PCR was used to evaluate swab specimens from 38 sequential patients with genital ulcer disease who received clinical diagnoses and syndromic treatment (7). These specimens were also tested for *H. ducreyi* by culture which was reported to be negative for all specimens tested. Of the 38 specimens, the multiplex PCR detected HSV in 31 specimens (81.6%) and HSV and *T. pallidum* in 1 specimen (2.3%) (coinfection). The test was negative in the remaining six specimens (15.8%). The clinical diagnoses corresponded poorly to the results of the multiplex PCR for chancroid and syphilis; none of the samples from the six suspected cases of chancroid and the one case of syphilis were positive for *H. ducreyi* and *T. pallidum*, respectively, by the multiplex PCR. Of the six clinically suspected cases of chancroid, the multiplex PCR was negative for all pathogens in one case and positive for HSV in the remaining five cases. Of 24 clinically suspected cases of genital herpes, 21 cases (87.5%) were confirmed by multiplex PCR.

Although clinical findings correlated poorly with multiplex PCR results, it is known that the clinical manifestations of all three infections can vary significantly (25). A few specimens produced negative results for which there may be several possible explanations, including ulcers due to trauma, low organism numbers, inadequate sampling techniques, and lesions due to other etiologic agents. These studies highlight the advantages of multiplex PCR over standard laboratory techniques and allow the detection of coinfection.

Ocular Infections

The benefits of PCR diagnostics over conventional techniques in the diagnosis of ocular infection are well documented for both the anterior and posterior segment of the eye (1, 24, 31, 80). The development and evaluation of multiplex PCR for detection of adenovirus, HSV, and *C. trachomatis* in cases of keratoconjunctivitis demonstrated the feasibility of simultaneous screening for these agents (49; A. C. Yeo, R. J. Cooper, D. J. Morris, and C. C. Storey, Abstr. 97th Gen. Meet. Am. Soc. Microbiol., abstr. C-416, 1997). These studies further highlight the difficulties of multiplex PCR and also provide substantial evidence for the importance of careful selection of oligonucleotide primers. In the study by Jackson et al. (49), a multiplex PCR was designed to detect adenovirus and HSV in eye swabs. The test produced results identical to those of virus isolation for 18 of 20 eye swabs (positive for adenovirus in five swabs, positive for HSV for five swabs and negative for adenovirus and HSV in eight swabs) but the remaining two specimens positive for adenovirus and HSV by virus isolation were negative by the multiplex PCR. However, the multiplex PCR proved superior to culture for the rapid diagnosis of viral keratoconjunctivitis. Replacement of the adenovirus primer pair to allow broader reactivity with adenovirus serotypes and inclusion of a primer pair targeting the cryptic plasmid of *C. trachomatis* (A. C. Yeo, R. J. Cooper, D. J. Morris, and C. C. Storey, Abstr. 97th Gen. Meet. Am. Soc. Microbiol., abstr. C-146, 1997) yielded a triplex multiplex PCR. The sensitivity of this adenovirus-HSV-*C. trachomatis* multiplex PCR in comparison to a uniplex PCR for the detection of adenovirus was 100%. However, the performance of the test to detect either HSV or *C. trachomatis* was relatively poor (69% compared to cell culture or 72% compared to an antigen detection technique). Selection of alternate HSV and *C. trachomatis* primer pairs allowed development of a multiplex PCR with identical sensitivity to that of uniplex PCRs for detection of each of the three targets.

Immunocompromised Patients

Due to the importance of HHV infections in immunocompromised patients, a number of authors have developed methods for the simultaneous detection of these viruses in various clinical specimens. Two different genes of CMV were detected with a single-step multiplex PCR in clinical specimens from renal transplant recipients and other CMV-seropositive patients (11). The test was sensitive and allowed monitoring of CMV infection by quantitation of CMV DNA. The latter was based on the assumption that samples with small amounts of viral DNA are more likely to lead to the amplification of only one of the two targets. Samples from which a single target is amplified contain on average sevenfold fewer viral genomes per 10^6 leukocytes than those from which both targets are amplified. This approach was evaluated on serial leukocyte DNA samples taken from 34 patients during the first 12 weeks post-renal transplantation, and it was concluded that findings of three consecutive tests in which both CMV targets were amplified were highly indicative of patients who had developed a very high load of CMV with a sensitivity of 100% and specificity of 88%. Thus, the protocol can be used for efficient identification of transplant recipients at risk of clinically significant infection. A similar PCR format was used to develop a multiplex PCR for amplification of four different regions of the CMV genome (61). The test sensitivity for detection of genomic cell DNA infected with CMV was similar to that obtained by each pair of the primers (uniplex PCRs). The test

proved to have a high diagnostic utility for detection of CMV variants with maximal sensitivity and specificity.

In heart transplant recipients a hot start nested multiplex PCR was used to evaluate the possible reactivation of HHV-6 and HHV-7 (67). The test was also coupled with CMV antigenemia assay and HSV isolation. The multiplex PCR was performed on buffy coat and plasma samples from the 21 recipients and on buffy coat samples from healthy blood donors. Whereas 12 of 21 (57.1%) heart transplant recipients showed CMV and/or HSV reactivation, HHV-6 was detected in 2 of 21 (9.5%) of the recipients and in 7 of 56 (12.5%) blood donors. HHV-7 DNA was detected in 13 of the 21 recipients (61.9%) and in 30 of 56 (56.6%) blood donors. One of the patients positive for HHV-6 and 10 of those positive for HHV-7 were positive for CMV. The clinical significance of these concurrent infections remains to be determined, but their detection further highlights the utility of multiplex PCR in investigation of transplant patients.

Detection and typing of all human lymphotropic herpesviruses (EBV, CMV, HHV-6 variants A and B, HHV-7, and HHV-8) utilizing primer pairs designed to amplify a highly conserved region within the DNA polymerase gene have also been described (77). The test also included an internal control (100 molecules of a cloned fragment of the porcine pseudorabies herpesvirus genome) for detection of false-negative results. The test revealed a sensitivity of 10 to 100 molecules of each virus DNA and produced a 100% correlation in a total of 35 well-characterized specimens in which one of these viruses was known to be present, including Kaposi's sarcoma skin lesions and serum, CSF, saliva, and urine samples.

Simultaneous detection of EBV and the protozoa *Toxoplasma gondii* in CSFs of AIDS patients with EBV-associated primary nervous system lymphoma and toxoplasma encephalitis has been described (87). This multiplex PCR detected EBV DNA in 9 of 14 patients with CNS lymphoma and in 2 of 38 patients without disease and *T. gondii* DNA in 8 of 8 patients with toxoplasma encephalitis but in none without toxoplasmosis.

The major problem with the use of PCR in specimens from immunocompromised patients is the relationship of positive results to clinical disease. This is particularly difficult with herpesviruses, because positive PCR results may not always be accompanied by signs or symptoms in the patient. This can leave the clinician in a dilemma of whether or not to treat. Two approaches have been used to address this problem. Quantitative PCR can be used to measure viral load with the expectation that a high viral load is likely to herald clinical disease. The other approach is to test sequential specimens and only recommend treatment if positive results persist rather than responding to what may be a transient reactivation of a herpesvirus. Multiplex PCR may be readily applied to the latter situation, but multiplex quantitative PCR remains a formidable technological challenge.

Other Applications

A number of studies have utilized multiplex PCRs for detection and differentiation of human retroviruses (45, 46, 99, 111). Four primer pairs were combined to detect the *gag* region of HIV type 1 (HIV-1), the *env* region of HIV-2, the *pol* region of human T-cell leukemia virus type 1 (HTLV-1), and the *tax* region of HTLV-2 (45). Amplicons were detected by liquid hybridization using ^{32}P-end-labeled oligonucleotides. In the evaluation of a serologically well-established panel of singly and dually infected individuals, the assay detected 21 of 22 HIV-1, 8 of 10 HIV-2, 8 of 8 HTLV-1, and 8 of 8 HTLV-2 infections. The test was as sensitive as uniplex PCRs and allowed the detection of coinfection. Sunzeri et al. (99) developed a multiplex PCR utilizing primer pairs targeting a portion of the *gag* region of HIV-1, the *pol* gene of HTLV-1 and -2, and a region of the HLA-DQ-α locus as an internal control. Products were analyzed by automated capillary DNA chromatography (products can also be separated and visualized using gel electrophoresis and ethidium bromide staining). The test detected as few as 1 to 10 infected cells (2 to 20 target sequences) and was as sensitive as uniplex PCRs.

Several studies have highlighted the potential use of PCR in screening donated blood for transfusion-transmitted viruses (48), but the need for multiple, discrete PCR assays for achieving this purpose has restricted such an application. Uniplex PCR would be an impractical approach for rapid screening of hundreds of specimens per day for a range of transfusion-transmitted viruses. Multiplex PCR presents a more practicable solution to the problem, and the methodology is sensitive enough to diagnose silent (serologically negative) carriers of viruses such as HIV, HTLV, and other nonretroviral transfusion-transmitted viruses, including hepatitis B virus (HBV), HCV, and CMV.

Multiplex PCR offers a cost-effective solution which, with refinement and full automation would allow screening of the donated blood supply. Indeed, a recent study (106) demonstrated the promise of multiplex PCR automation for both rapid and reliable screening of transfusion-transmitted viruses in donated blood and transplantable tissues. A hot start multiplex PCR was developed to identify and determine the abundance of HIV-1, HIV-2, and HTLV-1 and -2. Viral DNA sequences were amplified in a single reaction, and the resulting amplicons were detected in real time by the hybridization of four differently colored, amplicon-specific detector probes called molecular beacons present within the same reaction tube. The color of the fluorescence produced during the amplification process identified the retrovirus present in the sample, and correlation of the thermal cycles required with the intensity of each fluorescent signal developed provides an accurate measure of the number of virus sequences present in the original sample. The test had a sensitivity of 10 retroviral genomes in the presence of 100,000 copies of another retrovirus, and up to 96 samples can be analyzed in 3 h on a single plate. The test was also evaluated using 43 human blood samples known to contain human retroviruses and produced 100% agreement in 11 samples positive for HIV-1, 4 samples positive for HIV-2, 15 samples positive for HTLV-1, and 17 samples positive for HTLV-2, and the 10 control samples remained negative for all targets.

Multiplex PCRs for other transfusion-transmitted viruses have also appeared in the literature. A multiplex PCR which detects both HBV and HBC genomic sequences in serum samples has been developed (71). The test is carried out in two stages. HCV RNA is first reverse transcribed into cDNA, and both HCV cDNA and HBV DNA are then coamplified using primers that target conserved sequences from both viruses. The test was applied to sera from nine donors, of which seven were positive for HBsAg, anti-HBc, and anti-HCV; one was reactive for both anti-HCV and anti-HBc; and one was reactive for both HBsAg and anti-HBc. The multiplex PCR produced results confirming the presence of both HBV- and HCV-specific genomic sequences in eight of eight sera reactive for the serological markers of both viruses and also in a serum that was reactive for HBV markers only. The method was modified to include primers that target the HCV 5′ untranslated region and HBV pre-S and S region in a one-step multiplex PCR method. This modification allowed a simple simultaneous am-

plification of both viruses with 100% concordance with its respective uniplex PCRs (47).

HCV and GB virus type C (GBV-C)/HGV genomes in plasma samples from transfused subjects have also been amplified by a multiplex PCR (16). The test was evaluated retrospectively in 50 plasma samples in comparison with the results of serology. Of the 50 samples, 40 were positive for anti-HCV and 10 samples remained antibody negative. The multiplex PCR and the corresponding uniplex PCR produced identical results, being positive for HCV RNA in 32 samples of the 40 anti-HCV positive samples. GBV-C RNA was detected in 5 of the 32 HCV-positive samples (coinfection) and in 2 of 10 samples that were anti-HCV negative. More widespread application of multiplex PCR aimed at detecting transfusion-transmitted viruses and/or other pathogens will demand thorough optimization and full automation of procedures to avoid the production of both false-positive and false-negative results. The demands of transfusion medicine of high-speed, high-throughput screening will place great technical demands upon these procedures.

Multiplex PCR methodology has also proven to be a valuable tool for differentiation, subgrouping, subtyping, and genotyping of viruses (10, 29, 78, 84, 121). Differentiation of polioviruses from nonpoliovirus enteroviruses in both clinical (stool) and environmental (sewage) specimens has been feasible using RT-multiplex PCR (29). Stool or sewage specimens are first inoculated onto cell cultures in tubes, and after overnight incubation the cultures are subjected to RT-multiplex PCR. A primer pair detecting all enteroviruses in the presence of another two primer pairs specific for the polioviruses was tested. The enterovirus-specific primer pair generated a product size of 300 bp, and the poliovirus-specific primer pairs generated three different PCR products of 200, 600, and 1,000 bp, assuring easy identification on agarose gels. The result is interpreted as "nonpolio enterovirus" if only the enterovirus-specific PCR product (300 bp) is observed; as "poliovirus" if both the enterovirus-specific product (300 bp) and at least one of the polio-specific products are observed (200, 600, and 1,000 bp); or as "no enterovirus" if the multiplex PCR remains negative for all products. A total of 36 poliovirus strains produced the expected results by the multiplex PCR, consistent with polioviruses, and only one isolate (coxsackievirus A21) of 45 nonpoliovirus strains demonstrated a band with a poliovirus-specific primer pair. The protocol revealed a sensitivity of 3 PFU, could be interpreted within 24 h, and was highly insensitive to substances in the sample (stool and sewage) which inhibit cell culture isolation.

A multiplex RT-PCR that simultaneously identifies Sabin poliovirus types 1, 2, and 3 vaccine strains has been described (10). This hot start-based multiplex PCR was a modification of a procedure previously described (121). The assay utilized three serotype-specific primer sets that map to the region of the poliovirus genome encoding the amino terminus of the VP1 capsid protein, a region known for its heterogeneity among the three Sabin poliovirus serotypes. In a total of 195 stool samples collected from 26 vaccinees following administration of the first dose of the trivalent oral vaccine, the multiplex PCR was more sensitive than culture for the detection of poliovirus types 1, 2, and 3. The percentages of specimens positive by the multiplex PCR for serotypes 1, 2, and 3 were 67.2, 82.6, and 53.8%, respectively, compared to 55.4, 64.1, and 27.7% by virus isolation. The duration of recovery of positive samples by PCR varied according to serotype: 4 to 8 weeks for type 2 and 1 to 8 weeks for types 1 and 3, although poliovirus type 3 shedding ceased in approximately 70% of vaccinees within a week after immunization. This modified multiplex PCR allows direct characterization of the virus in stool specimens without cell culture—a process which may, through the selection of genetic variants, not accurately represent the virus population in the original specimens.

Human adenoviruses in clinical samples were detected with six primer pairs specific for all adenovirus subgenera (A to F) (78). Each primer pair consists of a primer derived from the subgenus-specific sequences and a primer that targets a conserved hexon region, obviating the need for restriction endonuclease analysis. The six subgenus-specific amplicons were distinguishable by agarose gel electrophoresis as products of 299, 465, 269, 331, 399, and 586 bp representing, respectively, those of adenovirus subgroup A to F. The test revealed a detection limit of a single copy of adenovirus DNA. The primer pairs produced 100% specificity when evaluated on 23 adenovirus prototypes, representing all six subgenera; on 9 intermediate strains from subgenera B and D; and on 16 subgenus C genome types. In clinical specimens, the test was positive for adenovirus in 26 of 65 stool specimens (4 samples belonging to subgroup A, 2 samples belonging to subgroup B, 6 samples belonging to subgroup C, 13 samples belonging to subgroup F, and 1 sample showing coinfection with subgroup C and F), in 13 of 23 eye specimens (1 sample belonging to subgroup B, 10 samples belonging to subgroup D, and 2 samples belonging to subgroup E), and 2 of 12 throat specimens (belonging to adenovirus subgroup B). Of significance, the test has clinical value as can be highlighted by its discrimination of adenovirus subgroup D, which causes the severe and highly contagious epidemic keratoconjunctivitis, from subgroup B and E adenoviruses, which may cause relatively mild ocular infections. The test could also facilitate the primary classification of unknown virus isolates.

Multiplex PCR has also proved to be a valuable tool for genotyping HBV strains (84). The first round of amplification utilizes a primer pair to amplify the entire pre-S region of the virus genome. Within the pre-S region nucleotide exchanges are observed that are partly correlated to the serological surface antigen subtypes. In the second round of amplification, five additional subtype-specific primers and two universal nongroup-specific primers are added to generate two to four DNA fragments of defined sizes indicative for the subtype. The method proved to be a useful epidemiological tool for studying HBV transmission and may be adapted to genomes of other infectious agents demonstrating a suitable degree of sequence variability.

Other applications of multiplex PCR include maximizing the inclusivity of PCR-based assays to detect viral strains (11, 51, 61, 93), exploring genetic reassortment among viruses (90), studying association of virus with disease (105), studying virus pathogenesis (6), exploring mechanisms of virus evasion and interference with the host's immune response (41), and facilitating the detection and characterization of viruses and other pathogens retrospectively in diverse archival specimens of limited volumes (2). In addition, the methodology has proved to be a powerful tool for characterization of nonhuman viruses (50, 70, 85, 104, 114, 116).

CONCLUSION AND PERSPECTIVES

Optimization of multiplex PCRs can prove difficult. A stepwise matrix-style approach may be followed; i.e., a number of optimal primer pairs are combined and the combination giving the best result is then chosen to be optimized or evaluated in a multiplex PCR format. Alterations of other PCR components over those usually described for most uniplex PCRs have rarely improved the efficiency of the test. Recent developments

in PCR technology, however, may facilitate the development of multiplex PCRs. The most appropriate of these seems to be the use of the nonmechanical hot start PCR (8, 53).

Thorough evaluation and validation of new multiplex PCR procedures is essential. The sensitivity and specificity must be thoroughly evaluated using standardized, purified nucleic acids. Where available, full use should be made of external quality control materials, and both external and internal quality controls must be rigorously applied. These must include the provision of both negative control specimens and, for each nucleic acid target, a positive control designed to ensure early signalling of any reduction in test sensitivity from assay to assay. As the number of microbial agents detectable by PCR increases, it will become highly desirable for practical purposes to achieve simultaneous detection of multiple agents that cause similar or identical clinical syndromes and/or share similar epidemiological features. In addition, attention should be given to primer pairs detecting multiple strains or types to ensure the identification of as many strains of the target species as possible. Where possible, robust (i.e., reliable in routine diagnostic settings) multiplex PCRs that do not use nested primers are preferable to avoid contamination (122), to rationalize use in routine settings, and to facilitate automation (110).

Commercial development of PCR has facilitated the widespread introduction of this procedure and improved both the reliability and ease of use of the technology. Commercially available applications of multiplex PCR are as yet in their infancy. While many commercially available PCRs include either internal control molecules or reporter molecules as internal standards within the test, the primer binding targets of both internal control and target nucleic acids are the same. Thus, although two molecules are amplified in the PCR, this is not true multiplexing. To date a multiplex PCR for *Neisseria gonorrhoea* and *C. trachomatis* represents the sole commercial multiplex PCR from Roche Diagnostics, and Argene Biosoft (Ariége, France) produces a multiplex PCR for detection of HHVs. Undoubtedly, many more commercial applications of multiplex PCR may be anticipated, and when coupled with developments in microelectronic detection devices (58) the prospect of extralaboratory "at-the-bedside" multiplex PCR testing may be envisaged. Given the advantages already demonstrated by the use of multiplex PCR along with the recent developments in this technology, future applications of PCR, when possible, should be aimed at constructing multiple detection systems in which a number of clinically and epidemiologically relevant pathogens (viruses, bacteria, parasites, and/or fungi) may be detected, characterized, and/or inevitably uncovered in a symptom- and/or system-specific manner.

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